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Sugar-Nucleotide Precursors of Arabinopyranosyl, Arabinofuranosyl, and Xylopyranosyl Residues in Spinach Polysaccharides[1]

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ABSTRACT

Cultured spinach (*Spinacia oleracea* L. cv Monstrous Viroflay) cells incorporated exogenous L-[^{3}H]arabinose sequentially into β-L-arabinopyranose-1-phosphate, uridine diphospho-β-L-arabinopyranose, uridine diphospho-α-D-xylopyranose and (in some experiments) α-D-xylopyranose-1-phosphate. The amount of ^{3}H in each of these compounds reached a plateau after a few minutes, and could be rapidly chased with nonradioactive L-arabinose, demonstrating rapid turnover. After a few minutes' lag, incorporation of ^{3}H into the arabinofuranosyl, arabinopyranosyl, and xylopyranosyl residues of polysaccharides was linear with respect to time. The kinetics of labeling were compatible with UDP-β-L-arabinopyranose and UDP-α-D-xylopyranose being the immediate precursors of arabinans (both the pyranose and the furanose residues) and xylans, respectively. No other radioactive nucleotides were formed; in particular, UDP-arabinofuranose was absent. There was no evidence for conversion of arabinopyranose to arabinofuranose within the polysaccharides, suggesting that this conversion occurs during polymer synthesis. The glycolipids detected showed too slow a turnover to be intermediates of pentosan synthesis.

The primary cell walls of higher plants contain polysaccharides rich in the pentoses L-arabinose and D-xylose. The sugar donors for the synthesis of the pentosans are generally believed to be UDP-β-L-Ara*p*[3] and UDP-α-D-Xyl*p* (1, 8, 21), and these have been successfully used as precursors *in vitro* (3, 8, 23, 24). However, it is not clear whether these are the only important NDP-pentoses present *in vivo*. The present paper describes a search for possible alternative structures. Possibilities for variation in the structure of NDP-pentoses exist at at least three levels. 1) Ring form of sugar. Most polymer-bound arabinose occurs in the furanose form (22), whereas only the pyranose form of UDP-arabinose has ever been synthesized enzymically *in vitro* by either of the available pathways: (a) UDP-Glc*p*A $\xrightarrow{\text{decarboxylase}}$ UDP-Xyl*p* $\xrightarrow{\text{epimerase}}$ UDP-Ara*p* (1), or (b) arabinose $\xrightarrow{\text{kinase}}$ Ara*p*-1-P $\xrightarrow{\text{pyrophosphorylase}}$ UDP-Ara*p* (21). To account for the Ara*p* → Ara*f* conversion during polymer synthesis, the possibility was considered that a further, unknown enzyme occurs *in vivo* to convert the UDP-Ara*p* into UDP-Ara*f*. This conversion would be analogous to the formation of UDP-Gal*f* as a polysaccharide precursor in fungi (13). 2) Nucleoside. The major nucleoside for both NDP-Ara and NDP-Xyl is uridine (8), but in addition ADP-Ara and GDP-Ara have been found in algae (17, 26), GDP-Ara in cotton (5), and GDP-Xyl in strawberry (16). The possible contribution of these nucleotides to polysaccharide synthesis in higher plants has not been assessed. 3) The possibility has been raised that UDP-oligosaccharides exist *in vivo*, including one containing glucose and arabinose (7); these could be potential precursors of certain sugar blocs which occur in polysaccharides and glycoproteins.

Here we report work to determine the extent to which such variation occurs in the NDP-pentoses that contribute to pentosan biosynthesis in cultured spinach cells. Analysis of nonradioactive NDP-pentoses requires the processing of large amounts of plant material, *e.g.* 1 kg of cambial scrapings (7), and it is generally unclear from the results whether the compounds are in a state of metabolic flux (and therefore possible precursors of the polymers) or metabolic end products with no precursor role. An alternative approach, adopted here, exploits the fact that cultured plant cells are able to incorporate exogenous L-arabinose, presumably via a scavenger pathway, very efficiently into their cell wall pentosans (18). The rate of incorporation into polymers becomes linear after a few minutes; therefore exogenous [^{3}H]arabinose should very quickly cause steady-state labeling of all obligatory intermediates.

MATERIALS AND METHODS

Radiochemicals. UDP-α-D-[U-^{14}C]Xyl*p* (154 mCi/mmol) was from New England Nuclear, West Germany. D-[U-^{14}C]Arabinose was from CEA, Gif-sur-Yvette, France. L-[1-^{3}H]Arabinose and D-[1-^{3}H]xylose were prepared at Amersham International, U. K., by catalytic exchange of about 30 mg of the pure sugar with 10 Ci of $^3H_2$ (method 'TL7'), and were purified chromatographically in system 1. The [^{3}H]arabinose (yield 1.6 Ci at about 8 Ci/mmol) was repurified by paper chromatography in system 4 immediately before each experiment.

Enzymes and Substrates. L-Arabinokinase was extracted from mung bean seedlings (20), and phosphorylations were carried out

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[3] Abbreviations: Ara*p*, arabinopyranose; Ara*f*, arabinofuranose; $M_{picrate}$, electrophoretic mobility relative to picrate; NDP, nucleoside diphosphate in which the identity of the nucleoside is not specified; PCV, packed cell volume; R_{Ara}, chromatographic mobility relative to arabinose; Xyl*p*, xylopyranose; Xyl*f*, xylofuranose.

with ATP and $MgCl_2$ in the presence of 40 mM KF (phosphatase inhibitor) as described (20). The product (β-L-Arap-1-P) was isolated by paper electrophoresis (pH 3.5). A crude mixture of UDP-sugar pyrophosphorylases was also extracted from mung bean seedlings (21) and the reaction of the sugar 1-phosphate with excess UTP in the presence of $MgCl_2$ and 40 mM KF was carried out as described (21). This enzyme mixture is specific for α-D-Xylp-1-P and β-L-Arap-1-P (20, 21). The products (UDP-α-D-Xylp and UDP-β-L-Arap) were isolated by paper electrophoresis (pH 3.5).

Acid phosphatase (grade I, from potato) was from Boehringer-Mannheim; it was used at 25 μg/ml in buffer *A* (0.2 M pyridine-acetate, pH 5.0), for 18 h at 20°C. Rattlesnake venom phosphodiesterase was from Boehringer-Mannheim, and was used at 10 μg/ml in buffer *B* (50 mM Tris-HCl, pH 8.9, containing 5 mM $CaCl_2$), for 2 h at 20°C. Fluoride was removed during the paper electrophoresis prior to treatment of samples with phosphatase or phosphodiesterase.

Authentic β-L-[^{3}H]Arap-1-P was prepared from L-[^{3}H]arabinose by use of L-arabinokinase (20), and authentic UDP-β-L-[^{3}H]Arap was obtained from the [^{3}H]ara-1-P by the action of UDP-sugar pyrophosphorylase (21). Yields were about 95% for the Ara-1-P and about 85% for the UDP-Ara. The products were purified by paper electrophoresis (pH 3.5) followed by paper chromatography (systems 2 and 3). Authentic α-D-[^{14}C]Xylp-1-P was prepared from UDP-α-D-[^{14}C]Xyl by the action of phosphodiesterase.

Extractions. Sugar-nucleotides and -phosphates and glycolipids were routinely extracted from cultured spinach cells as follows. A known fresh weight of cells (calculated from the measured fresh weight of an identical nonradioactive aliquot) was quickly collected on muslin in a Hartley funnel under suction, KF (400 mM) was added to a final concentration of 40 mM, and the cell slurry was transferred into a volume of ice-cold $CHCl_3$:methanol (1:1, v/v) calculated to give a final solvent composition of $CHCl_3$:methanol:H_2O (10:10:3, v/v), taking the fresh weight of the cells as H_2O. These operations took about 10 s. The suspension was stirred magnetically in a glass-stoppered tube at 4°C for 30 min, and the cell residue was removed by centrifugation. One sample of the extract was electrophoresed immediately (paper electrophoresis, pH 3.5) for analysis of the nucleotides and phosphates. For analysis of the lipids, a further sample was subjected to descending paper chromatography overnight in ethanol:H_2O (1:9, v/v) to remove nonlipid material, and then the lipid at the origin was eluted in $CHCl_3$:methanol:H_2O (10:10:3, v/v). Lipids were analyzed (a) by TLC or (b) by paper chromatography on DEAE-paper. The cell residue was washed with 80% ethanol until the washings were no longer radioactive, the residue was dried, and the polysaccharides were hydrolyzed with acid. The ethanol washings were pooled with the $CHCl_3$:methanol:H_2O extract, and analyzed for oligosaccharides by two-dimensional paper electrophoresis/chromatography (pH 3.5 and system 6).

The $CHCl_3$:methanol:H_2O- and 80% ethanol-insoluble polymer residue was fractionated as follows. Proteins and glycoproteins not covalently bound in the cell wall were extracted in phenol:acetic acid:H_2O (2:1:1, w/v/v) with stirring at 30°C for 16 h, and precipitated from the solution by addition of 0.02 volumes 10% aqueous $HCOONH_4$ plus 5 volumes acetone. The polysaccharide-rich material, insoluble in phenol:acetic acid:H_2O, was washed in acetone and dried, and the pectins were solubilized from it by nine extractions in 2% Na-hexametaphosphate adjusted to pH 3.7 with HCl, each at 100°C for about 8 h. The pectic extracts were pooled, dialyzed against water, and fractionated into neutral and acidic components by paper electrophoresis (pH 6.5). The material insoluble in hexametaphosphate was washed in water and hemicelluloses were extracted in degassed 17.4% NaOH containing 4% H_3BO_3 at 20°C for 16 h *in vacuo* (29). The hemicellulosic extract was neutralized and dialyzed.

Acid Hydrolyses. Complete hydrolysis of polysaccharides in 72% and 3% H_2SO_4 was performed by the method of Saemen (9). Selective hydrolysis of glycofuranosyl linkages was achieved in 30 mM oxalic acid at 100°C for 3 h; this treatment liberated approximately 80% of the arabinose from spinach cell walls, but little of the other sugars. A small proportion of the Arap linkages would also be hydrolyzed under these conditions (11). The oxalate was precipitated by dropwise addition of 0.2 M $Ba(OH)_2$ to the end point of bromophenol blue. Glycosyl phosphate esters were hydrolyzed with 0.01 M HCl at 100°C for 20 min, and the HCl was removed *in vacuo* at room temperature.

Reactions of Carbohydrates. Reduction of partial hydrolysates (the whole suspension, without removal of oxalate) was achieved with excess 0.25 M KBH_4 in 0.25 M NH_4OH at 20°C for 2 h. The reaction was stopped by addition of acetic acid to pH 5. The soluble components were subjected to column chromatography on Zeo-Carb to remove K^+ and NH_4^+; boric and acetic acids were removed by co-evaporation with methanol (10). The residue was dissolved in H_2O, recombined with the insoluble wall material, and completely hydrolyzed.

Chromatography and Electrophoresis. Unless otherwise stated, paper chromatography was performed on Whatman No. 1 paper, by the descending method, with the following solvents (all compositions are by volume): (1) ethyl acetate:pyridine:H_2O (8:2:1), (2) propan-1-ol:0.2 M morpholinium borate at pH 8.6 in 0.01% EDTA (13:7), (3) ethanol:1 M ammonium acetate at pH 3.8 (5:2), (4) butan-1-ol:acetic acid:H_2O (15:3:5), (5) butanone:acetic acid:saturated aqueous H_3BO_3 (9:1:1), (6) ethyl acetate:pyridine:H_2O (10:4:3). TLC was on silica gel in $CHCl_3$:methanol:H_2O (64:25:4). Paper chromatography on Whatman DEAE-cellulose paper was in H_2O-saturated butan-1-ol.

High-voltage paper electrophoresis of phosphates and nucleotides was performed on Whatman No. 1 paper, with white spirit as coolant (15–25°C). The buffer was 5% acetic acid adjusted to pH 3.5 with pyridine. The effective paper length was about 40 cm, and a potential of 5 kv was maintained for 30 min, cathode at the top. Paper electrophoresis of pectins was on Whatman GF/A glass fiber paper in 10% pyridine, adjusted to pH 6.5 with acetic acid and containing 10 mM Na_2EDTA, and 1 kv for 90 min, otherwise as above. Carbohydrates were located on the GF/A by reaction with sulphonated α-naphthol in ethanol:H_2SO_4 (28).

Determination of Radioactivity. Strips from chromatograms and electrophoretograms were submerged in 0.5% PPO in toluene, and assayed for ^{3}H at about 3% efficiency by liquid scintillation counting.

Tissue Culture. A green suspension culture of spinach (*Spinacia oleracea* L. cv Monstrous Viroflay) was maintained as previously described (10) in a medium containing 1% sucrose as sole organic constituent, and subcultured every 2 weeks by about 8-fold dilution. Growth was exponential between days 2 and 7 after subculture, and cells in this stage were used throughout the present work. Cell density was measured as PCV (12). For incubations with radioactive substrates, the PCV was usually increased by removal of some of the medium.

RESULTS

Incorporation of [^{3}H]Arabinose into Spinach Polymers. A spinach suspension culture (4 d) (100 ml; PCV adjusted to 80 μl/ml) was treated with L-[^{3}H]arabinose (100 μCi) for 8 h. A portion of the ethanol-insoluble residue was totally hydrolyzed and analyzed for radioactive sugars by paper chromatography in system 1. The products were [^{3}H]arabinose (74%) and [^{3}H]xylose (25%). Radioactive ribose, hexoses, deoxyhexoses, and uronic acids

could not be detected. Selective hydrolysis of furanose units by oxalic acid gave 80% of the arabinose, but less than 2% of the xylose, demonstrating a predominance of Ara*f* and Xyl*p* residues in the polymers. The isolated [^{3}H]arabinose could be completely phosphorylated by use of mung bean L-arabinokinase (10), showing that it was still the L-isomer. A sample of D-[^{14}C]arabinose could not be phosphorylated by this method. These observations were verified on several occasions, although the [^{3}H]Ara:[^{3}H]Xyl ratio varied somewhat.

Upon fractionation of the ethanol-insoluble material, ^{3}H was found in the proteins (yielding principally [^{3}H]Ara on H_2SO_4 hydrolysis), the pectinic acids ([^{3}H]Ara:[^{3}H]Xyl = 98.6:1.4), the neutral pectins ([^{3}H]Ara:[^{3}H]Xyl = 97.5:2.5), and the hemicelluloses ([^{3}H]Ara:[^{3}H]Xyl = 12:88). Within the pectic fraction, the acidic:neutral ratio increased with time (Fig. 1). The neutral [^{3}H]pectin constituted 49%, 25%, and 22.5% of the total [^{3}H] pectin after 4, 17, and 65 min incubation with [^{3}H]arabinose, respectively. This observation supports the conclusion of Stoddart and Northcote (28) that the neutral side chains of pectinic acid are synthesized free and later transferred to an acidic pectic backbone.

In parallel experiments, D-[^{3}H]xylose was incorporated into spinach polysaccharides at extremely low rates.

Optimization of Extraction of Nucleotides. A spinach culture (3 d) (6 ml; PCV adjusted to 130 μl/ml) was incubated for 12 min with L-[^{3}H]arabinose (250 μCi). The cells were quickly transferred into $CHCl_3$:methanol:H_2O:KF and stirred at 4°C, and aliquots (20 μl) of the cell-free extract were taken at intervals for paper electrophoresis (pH 3.5). The radioactivity coelectrophoresis with authentic UDP-Ara*p* was estimated: extraction of this putative UDP-[^{3}H]pentose pool was rapid, and the extract was reasonably stable at 4°C in the presence of the cell residue for at least 2 h. Maximal yield was achieved with about 30 min extraction. Subsequent addition of HCOOH (0.1 volume) (2) extracted no further UDP-[^{3}H]pentose. A 30-min treatment with the neutral $CHCl_3$:methanol:H_2O:KF solvent was therefore chosen for routine extractions.

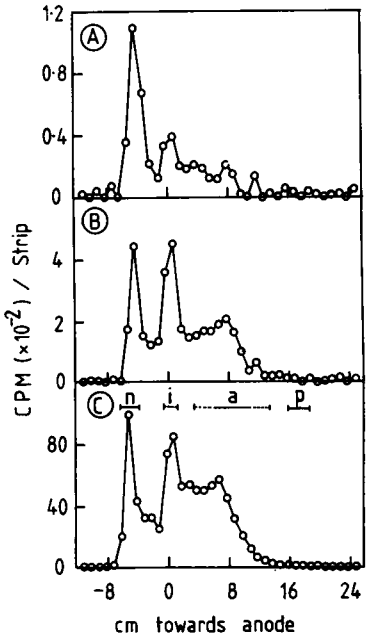

FIG. 1. Paper electrophoresis of pectic material from spinach cells incubated with [^{3}H]arabinose for A, 4 min; B, 17 min; or C, 65 min. Following electrophoresis at 1 kv for 90 min, the glass fiber paper was cut into 1-cm strips, which were assayed for radioactivity. A background value (65 cpm) has been subtracted from all the data. The strips from C were then washed with toluene, dried, and stained with sulfonated α-naphthol, revealing 3 carbohydrate-rich zones: *n* (neutral, moving slightly toward the cathode owing to electroendo-osmosis), *i* (immobile, probably acidic), and *a* (acidic). The position of a picric acid marker, *p*, is also shown.

Structural Analysis of Rapidly Labeled Phosphates and Nucleotides. A larger sample (100 μl) of the radioactive $CHCl_3$:methanol:H_2O:KF solution, obtained as above using 30 min extraction, was subjected to paper electrophoresis (pH 3.5), and a complete ^{3}H profile was obtained (Fig. 2). Three peaks of ^{3}H were found, corresponding in mobility with authentic arabinose ($M_{picrate}$ = 0.0), Ara*p*-1-P ($M_{picrate}$ = 1.0), and UDP-Ara*p* ($M_{picrate}$ = 1.1), respectively. The two acidic peaks were eluted with water and samples were subjected to hydrolysis with phosphatase, phosphodiesterase, and 0.01 M HCl. The products were analyzed by paper chromatography (system 1) and paper electrophoresis (pH 3.5) (Table I).

The radioactive material of $M_{picrate}$ = 1.0 was susceptible to phosphatase but resistant to phosphodiesterase and was therefore probably [^{3}H]pentose-P. Its instability in 0.01 M HCl shows that the phosphate was linked at C-1. The products of phosphatase and HCl digestion were [^{3}H]arabinose (82%) and [^{3}H]xylose (14%); and no radioactive ribose, hexoses, deoxyhexoses, uronic acids, or oligosaccharides could be detected. The material of $M_{picrate}$ = 1.0 is concluded to be a mixture of Ara-1-P and Xyl-1-P. Paper chromatography in system 2 (Fig. 3) showed that all the Ara-1-P was in the pyranose form (1), and rephosphorylation of its HCl-hydrolysis product with mung bean L-arabinokinase (20) established that it was all the L-isomer. In the presence of UTP, a pyrophosphorylase preparation from mung bean converted the spinach [^{3}H]Xyl-1-P and [^{3}H]Ara-1-P into UDP-[^{3}H]Xyl and UDP-[^{3}H]Ara, showing that the spinach compounds were α-D-[^{3}H]Xyl*p*-1-P and β-L-[^{3}H]Ara*p*-1-P, respectively (20, 21).

The radioactive material of $M_{picrate}$ = 1.1 also yielded [^{3}H] arabinose and [^{3}H]xylose upon treatment with 0.01 M HCl, indicating a phosphate linkage at C-1, but was essentially insensitive to phosphatase (Table I). Phosphodiesterase, in contrast, converted it to a product of $M_{picrate}$ = 1.0 which was indistinguishable from the β-L-[^{3}H]Ara*p*-1-P and α-D-[^{3}H]Xyl*p*-1-P described

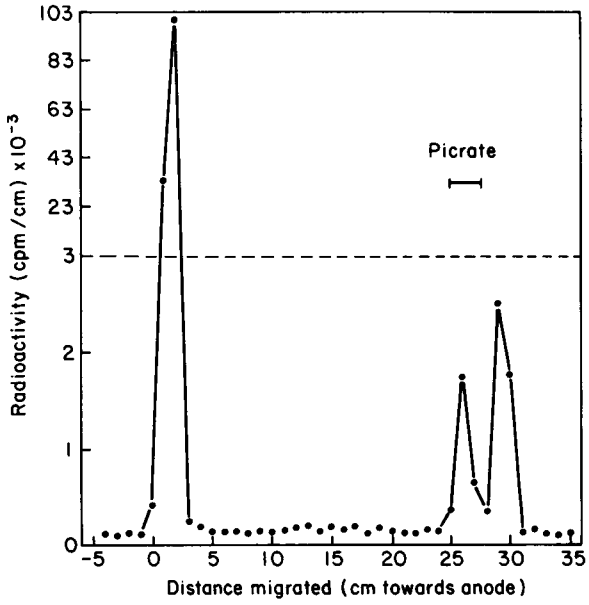

FIG. 2. Paper electrophoretogram at pH 3.5 of the material extracted in 30 min at 4°C by $CHCl_3$:methanol:H_2O (10:10:3 by vol) containing 5.22 mM KF, from cells incubated for 12 min with 41.7 μCi/ml L-[1-^{3}H] arabinose. The paper was cut into 1-cm strips, which were assayed for radioactivity.

Table I. *Hydrolysis Products of Acidic Metabolites of [^{3}H]Arabinose*

A spinach culture was fed [^{3}H]arabinose for 12 min, and the acidic metabolites were eluted from a paper electrophoretogram (pH 3.5), hydrolyzed and reanalyzed.

$M_{picrate}$ of Material Hydrolyzed	Method of Hydrolysis[a]	Composition of Hydrolysis Products[b] Reanalyzed							
		By chromatography in system 1				By electrophoresis at pH 3.5			
		Imm	Ara	Xyl	Other	Neut	Phos	Nucl	Other
						%			
1.0	HCl	3	82	14	1	97	0	0	3
	Control	97	1	1	1	0	98	1	1
	P-ase	0	85	15	0	99	0	1	0
	Control	98	1	0	1	1	99	0	1
	PD-ase	94	4	1	1	5	93	1	1
	Control	96	1	1	2	2	97	0	1
1.1	HCl	2	67	27	4	96	0	0	4
	Control	99	0	0	1	2	3	94	1
	P-ase	95	3	1	1	10	4	85	1
	Control	99	0	0	1	2	2	96	0
	PD-ase	92	6	1	1	8	87	4	1
	Control	96	2	1	1	3	3	93	1

[a] Controls for the HCl, P-ase, and PD-ase hydrolyses were treated for an equal length of time at 20°C with H_2O, buffer *A* and buffer *B*, respectively. P-ase, phosphatase; PD-ase, phosphodiesterase.

[b] Imm, chromatographically immobile; Neut, neutral; Phos, co-electrophoresing with authentic Ara*p*-1-P ($M_{picrate}$ = 1.0); Nucl, co-electrophoresing with authentic UDP-β-L-Ara*p* ($M_{picrate}$ = 1.1).

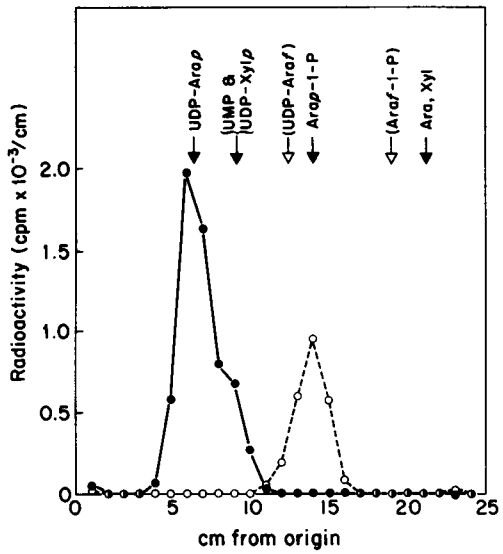

FIG. 3. Paper chromatogram (system 2) of material eluted from the electrophoretogram illustrated in Fig. 2. O---O, Pentose-phosphates; ●——●, UDP-pentoses. The positions of authentic markers are shown by solid arrows; the positions of Ara*f*-1-P and UDP-Ara*f* (open arrows) are calculated from the R_{UMP} values quoted by Aspinall *et al.* (1).

above. This shows that the material was composed of NDP-β-L-[^{3}H]Ara*p* and NDP-α-D-[^{3}H]Xyl*p*. As a direct test for the Ara*f* derivative, a sample was run by paper chromatography in system 2 (Fig. 3): this gave two peaks of ^{3}H at positions corresponding with UDP-β-L-Ara*p* (R_{UMP} 0.7) and UDP-α-D-Xyl*p* (R_{UMP} 1.0) (incompletely resolved); no radioactive material ran at R_{UMP} 1.4, the mobility reported for UDP-β-L-Ara*f* (1). The products of hydrolysis with 0.01 M HCl or with phosphodiesterase plus phosphatase were only arabinose (71%) and xylose (29%); ribose, hexoses, deoxyhexoses, uronic acids, and oligosaccharides were undetectable. Paper electrophoresis (pH 3.5) is known to resolve families of nucleotides differing in the nucleoside moiety (2). Therefore, the fact that all the radioactive nucleotide from spinach ran on paper electrophoresis (pH 3.5) as a single spot coincident with authentic UDP-Ara shows that uridine was the only nucleoside present. This conclusion was confirmed by the finding of only a single radioactive spot of NDP-Ara, which co-chromatographed with authentic UDP-Ara, upon paper chromatography in system 3, which has been shown to separate UDP-Gal from ADP-Gal, CDP-Gal, GDP-Gal, and TDP-Gal (25).

$CHCl_3$:methanol:H_2O:KF extracts obtained by exposure of the radioactive cells to the solvent for various lengths of time (3–120 min) were qualitatively and quantitatively very similar to the 30-min extracts described above. However, all the above results were obtained with freshly isolated nucleotide samples. Upon storage at −20°C, the nucleotides were slowly converted to compounds with the properties of pentose-1,2-cyclic phosphates (27) at R_{UMP} (system 2) about 1.6 to 1.9.

Kinetics of Labeling of Pentose 1-Phosphates, UDP-Pentoses, and Pentosans. [^{3}H]Arabinose (0.7 mCi) was supplied to a spinach culture (2 d) (10 ml; PCV adjusted to 150 μl/ml) and cell samples were withdrawn at intervals and extracted with $CHCl_3$:methanol:H_2O:KF. A portion of each extract was run by paper electrophoresis (pH 3.5) to separate the pentose 1-phosphates from the pentose-nucleotides. These were hydrolyzed separately in HCl and the resultant [^{3}H]pentoses were separated by paper chromatography in system 1 and assayed for radioactivity. In this way, the radioactivity in the four compounds Ara-1-P, Xyl-1-P, UDP-Ara, and UDP-Xyl was deduced for each time point. In addition, the radioactivity in the $CHCl_3$:methanol:H_2O:KF-insoluble (polymer-rich) fraction was assayed.

After a short lag, ^{3}H was incorporated into polymer, linearly for the duration of the experiment (and, in longer experiments at lower cell density, for 3–4 d). The linear rate was 6.2% of the supplied [^{3}H]arabinose h^{-1}. In contrast, all four low mol wt compounds quickly stopped net accumulation of ^{3}H, consistent with their being rapidly turned over. The kinetics (Fig. 4) were consistent with the sequence of labeling predicted from the pathway arabinose $\xrightarrow{\text{arabinokinase}}$ Ara-1-P $\xrightarrow{\text{UDP-Ara pyrophosphorylase}}$ UDP-Ara $\xrightarrow{\text{UDP-Xyl 4-epimerase}}$ UDP-Xyl → Xyl-1-P. Similar re-

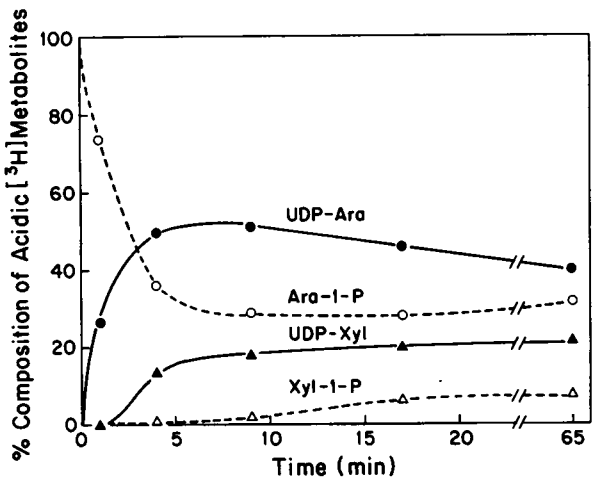

FIG. 4. Kinetics of labeling of pentose 1-phosphates and UDP-pentoses. A spinach culture (PCV = 150 μl/ml) was fed [^{3}H]arabinose, and at intervals, samples were extracted with $CHCl_3$:methanol:H_2O containing 5.22 mM KF. Aliquots (20 μl) of these extracts were analyzed for low mol wt acidic metabolites. For each time point, the total ^{3}H in the four compounds (Ara-1-P + Xyl-1-P + UDP-Ara + UDP-Xyl) was taken as 100%. (Absolute amounts of ^{3}H [counted on paper] corresponding to each of these 100% values were: 246, 2020, 5038, 6694, and 6127 cpm for the 1-, 4-, 9-, 17-, and 65-min samples respectively.)

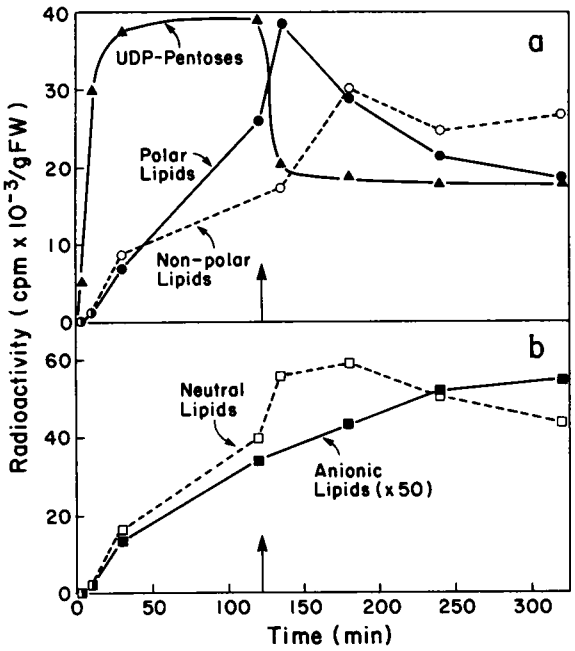

FIG. 5. Pulse-chase kinetics of labeling of glycolipids. A spinach culture (PCV = 250 μl/ml) was fed [^{3}H]arabinose followed after 120 min by 2 mM nonradioactive arabinose (↑). At intervals, samples were extracted and the washed lipid fraction was analyzed (a) by TLC on Si-gel, and (b) by paper chromatography on DEAE-paper in BuOH. For comparison, the kinetics of labeling of UDP-Ara + UDP-Xyl (▲——▲) are shown.

sults were obtained in several repeats of this experiment, except that [^{3}H]Xyl-1-P was not always detected. Rapid turnover of the nucleotides was confirmed in a pulse-chase experiment (Fig. 5a).[4]

Kinetics of Labeling of Glycolipids. The mixture $CHCl_3$:methanol:H_2O (10:10:3) is a recommended solvent for glycolipids, including those of the dolichol type (4): such lipids have been proposed as possible precursors of various glycoproteins and polysaccharides (15). To test for their possible contribution to pentosan synthesis, a spinach culture (PCV 250 μl/ml) was fed [^{3}H]arabinose (49 μCi/ml; total exogenous arabinose concentration about 6 μM), and samples were taken at intervals and extracted as before. After 120 min, solid L-arabinose was added to 2 mM as a cold chase, and further samples were taken.

Analysis of the lipid fraction by TLC showed two peaks of radioactivity (approximately equal) at R_F 0.65 and 0.87, designated polar and nonpolar lipids, respectively. Neither class of lipid stopped accumulating ^{3}H during the first 120 min of labeling (Fig. 5a), showing that they were probably not precursors of the polysaccharides (compare Fig. 6b). During the chase, the nonpolar lipids continued to accumulate some ^{3}H; the polar species tended to lose radioactivity (indicating turnover), but not rapidly enough to be precursors of major polysaccharides (Fig. 5a).

Samples of the lipid fraction were also analyzed by chromatography on DEAE-paper in BuOH. By this method, 2 peaks of ^{3}H were found (R_F 0.0 and 0.8, the latter co-chromatographing with Chl). The immobile (anionic) peak, which would include any dolichol-phosphate or -pyrophosphate sugars, accounted for about 2% of the total radioactive lipid. Again, it did not show labeling kinetics consistent with its possible role in polysaccharide synthesis (Fig. 5b).

Kinetics of Labeling of Ara*p*, Ara*f*, and Xyl*p* Residues of Polymers. A few Ara*p* units do exist in mature pectins (11, 22). It has been speculated (6, 8) that, since UDP-Ara*f* is unknown in plants, the Ara*p* → Ara*f* transition may occur within the polymer. The following experiment was designed to test this hypothesis.

Spinach cells (3 d) (PCV adjusted to 65 μl/ml) were incubated with [^{3}H]arabinose as described in Figure 4. The polymer fraction was treated with oxalic acid to hydrolyze selectively the furanosyl linkages, and the exposed reducing termini (including released mono- and oligosaccharides) were reduced to alditols with KBH_4. Hydrolysis was then completed with H_2SO_4. In this way, most of the Ara*f* and Xyl*f* residues would be released as arabinitol and xylitol, respectively, whereas most of the Ara*p* and Xyl*p* residues would be released as arabinose and xylose. [^{3}H]Xylitol was undetectable, but the other three products were separated by paper chromatography (system 5) and assayed for ^{3}H (Fig. 6a).

Ara (*f* and *p*) residues were labeled before Xyl*p* residues, as expected from the fact that UDP-Ara was labeled before UDP-Xyl. However, within the time limits of sampling, the Ara*f* and Ara*p* residues became labeled simultaneously. The proportion of [^{3}H]Ara*p* residues in polymers showed no tendency to decline. These observations support the view that the Ara*p* → Ara*f* transition occurs before or during polymer synthesis rather than after. Note that the first sample analyzed for Ara*p*, Ara*f*, and Xyl*p* was taken 7.5 min after the addition of [^{3}H]arabinose, but that this was only about 2.5 min after the commencement of polymer labeling.

The results (Fig. 6b) also show that polymer-bound Ara and Xyl started to accumulate ^{3}H as soon as their respective UDP derivatives became labeled. This suggests that the UDP-sugars are the direct precursors of the pentosans.

Incorporation of [^{3}H]Arabinose into Oligosaccharide-like Compounds. It could be argued that much of the early-synthesized [^{3}H]arabinan would be of low mol wt and therefore soluble in $CHCl_3$:methanol:H_2O or 80% ethanol. If so, growing oligosaccharides rich in Ara*p* might have been overlooked in the Ara*p*/

[4] Addition of a 2 mM nonradioactive arabinose chase (Fig. 5a) inhibited by 83% the incorporation of [^{3}H]arabinose into polymers, whereas it decreased the steady-state amount of ^{3}H in the UDP-pentose pool by only 55%. This may indicate a swelling, by high concentrations of exogenous arabinose, of the absolute pool size of UDP-pentose. Exogenous galactose is known to swell the pool of UDP-galactose (19).

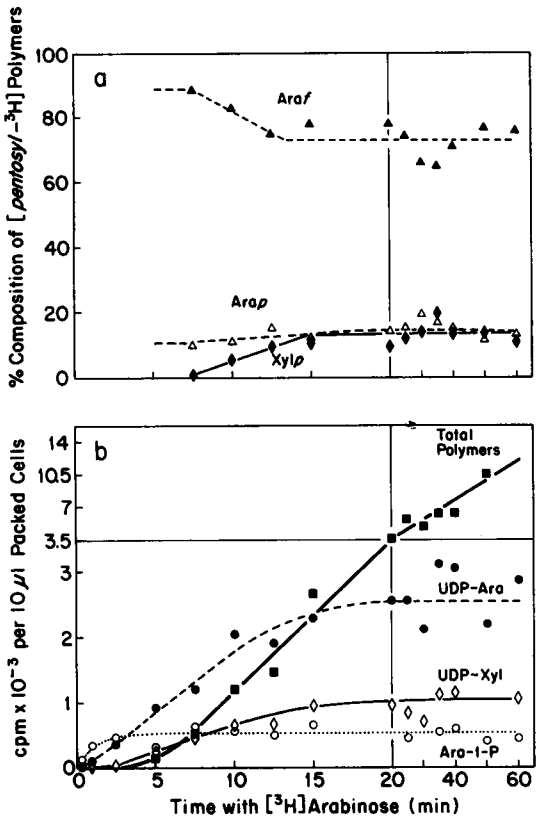

FIG. 6. Kinetics of labeling of Ara*p*, Ara*f*, and Xyl*p* residues of polymers. A spinach culture (PCV = 65 µl/ml) was fed [^{3}H]arabinose, and samples were extracted at intervals. (a) The polymeric material, insoluble in $CHCl_3$:methanol:H_2O and 80% ethanol, was analyzed for Ara*f*, Ara*p*, and Xyl*p* residues. (b) The extracted low mol wt components were analyzed as before; Xyl-1-P was undetectable in this experiment. The incorporation of ^{3}H into the total polymer fraction is shown in (b) for comparison. Note the discontinuities in the abscissa (a and b) and the ordinate (b).

Ara*f* analysis described above. To investigate this possibility, the electrophoretically neutral fraction of the $CHCl_3$:methanol:H_2O and ethanol extracts were analyzed further by paper chromatography (system 6). A paper electrophoretogram that had yielded 40,600 cpm of UDP-pentose (the 17-min sample in Fig. 4) gave the following neutral compounds: unmetabolized [^{3}H]arabinose (543,000 cpm), essentially no free [^{3}H]xylose (R_{Ara} = 1.24; less than 500 cpm; no discrete peak or shoulder), and traces of [^{3}H] oligosaccharide-like material (R_{Ara} = 0.0–0.9; 31,200 cpm; streak). The ^{3}H in the oligosaccharide-like fraction was substantially higher after 65 min labeling (12,900 cpm) than after 17 min; this would not be expected if the oligosaccharide-like compounds were precursors of the organic solvent-insoluble polymers.

DISCUSSION

Feeding of exogenous [^{3}H]arabinose to cultured spinach cells resulted in a linear rate of labeling of polymer-bound Ara and Xyl residues, after a lag of about 5 to 10 min. Therefore, all obligatory intermediates of polymer synthesis would become labeled to a plateau within 5 to 10 min. The only pentose-nucleotides detected with this pattern of labeling were UDP-β-L-Ara*p* and UDP-α-D-Xyl*p*. Others, if they exist at all, did not become ^{3}H-labeled fast enough to be regarded as intermediates of pentosan biosynthesis *in vivo*.

The arabinofuranosyl-phosphate linkage is highly acid-labile (Ara*f*-1-P is 47% hydrolyzed at pH 2 and room temperature in 4 h, compared with 7% for Ara*p*-1-P [30]). It seems unlikely that much UDP-Ara*f* would have been lost even under the most severely acidic conditions used in the present work (30-min paper electrophoresis at pH 3.5 and room temperature). It is also unlikely that sugar-nucleotides containing nucleosides other than uridine would be so much more labile as to be completely lost. There is also no reason to believe that linkages of oligosaccharide-nucleotides should be particularly unstable: the glycosidic linkages of oligosaccharides are considerably more resistant to hydrolysis than are comparable glycosyl-phosphate bonds. Direct evidence that no breakdown of xylose-nucleotides to free xylose was occurring comes from the observation that negligible free [^{3}H]xylose was present on two-dimensional paper electrophoretic/chromatographic analyses of spinach extracts that yielded 11,800 cpm of UDP-[^{3}H]Xyl. Therefore, it is concluded that UDP-β-L-Ara*p* and UDP-α-D-Xyl*p* are the only significant pentose-nucleotides in spinach cells. This finding vindicates the use of these compounds as radioactive precursors for *in vitro* studies of polysaccharide biosynthesis.

The kinetic data suggest that UDP-β-L-Ara*p* and UDP-α-D-Xyl*p* are also the immediate precursors of the polymers. If any other intermediates are involved, they must turn over very much more rapidly than the nucleotides. No evidence for such intermediates could be obtained from direct analysis of the radioactive lipids.

Most of the Ara residues in the primary cell walls of dicotyledons are present in pectins, and are mainly in the α-L-furanose form. Inversion of the anomeric configuration is a consequence of most glycosyltransferase reactions, and is compatible with the proposed single-step sugar transfer from UDP-Ara (β in the nucleotide → α in pectin), rather than one involving a lipid intermediate. The ring contraction (Ara*p* in the nucleotide → Ara*f* in pectin) remains unexplained; nevertheless, the present work shows that ring contraction occurs either simultaneously with the glycosyl transfer or within just a few seconds following transfer.

The sequential labeling of Ara-1-P, UDP-Ara, UDP-Xyl, and Xyl-1-P is consistent with the expected pathway. In particular, the fact that [^{3}H]Xyl-1-P only appeared after UDP-Xyl had become labeled, shows that 4-epimerization occurs exclusively at the sugar-nucleotide level, and not at the sugar 1-phosphate level. This has not previously been investigated for pentoses *in vivo*.

In some experiments, [^{3}H]Xyl-1-P was detected. This was not a decomposition product formed artifactually from UDP-Xyl during extraction since the kinetics of labeling of Xyl-1-P and UDP-Xyl were different. In particular, the early time points (Fig. 4) showed UDP-[^{3}H]Xyl but no [^{3}H]Xyl-1-P. The kinetics indicate that UDP-[^{3}H]Xyl was the precursor of [^{3}H]Xyl-1-P. The mechanism of formation of Xyl-1-P from UDP-Xyl *in vivo* is unclear. It could be by reaction with PP_i under catalysis of UDP-Xyl pyrophosphorylase, or with P_i under catalysis of a phosphorylase (analogous to the recently discovered UDP-glucose phosphorylase [14]), or by phosphodiesterase-catalyzed hydrolysis, or any combination. Indirect conversion of UDP-Xyl to Xyl-1-P via free xylose is unlikely because: (a) free [^{3}H]xylose could not be detected in these experiments, (b) all known xylokinases form Xyl-5-P, not Xyl-1-P, and (c) exogenous [^{3}H]xylose was very slowly metabolized. Whatever its origin, the [^{3}H]Xyl-1-P itself was clearly undergoing rapid turnover and, since neither [^{3}H] xylose nor [^{3}H]pentose-5-Ps could be detected, it is likely that the fate of the Xyl-1-P was reconversion to UDP-Xyl by the action of UDP-Xyl pyrophosphorylase. Thus, the function of UDP-Xyl pyrophosphorylase and related pyrophosphorylases is probably the salvaging of sugar-phosphate units lost by cleavage of sugar nucleotides.

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